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BiP Acts as a Molecular Ratchet during Posttranslational Transport of Prepro-α Factor across the ER Membrane

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Summary

We have addressed the mechanism by which proteins are posttranslationally transported across the membrane of the yeast endoplasmic reticulum (ER). We demonstrate that BiP (Kar2p), a member of the Hsp70 family resident in the ER lumen, acts as a molecular ratchet during translocation of the secretory protein prepro-α factor through the channel formed by the Sec complex. Multiple BiP molecules associate with each translocation substrate following interaction with the J domain of the Sec63p component of the Sec complex. Bound BiP minimizes passive backward movements of the substrate through the channel, and BiP's subsequent dissociation results in a free polypeptide in the ER lumen. Antibodies against the substrate can replace BiP, indicating that a Brownian ratchet is sufficient to achieve translocation.

Introduction

Proteins are transported across the membrane of the endoplasmic reticulum (ER) through a channel whose central component is the heterotrimeric Sec61p complex. Transport through the channel can occur either co- or posttranslationally. In the cotranslational pathway, directionality of transport is determined by the binding of the translating ribosome to the Sec61p complex. The channels in the ribosome and the membrane are aligned, and the luminal end of the channel is therefore the only exit site for the elongating polypeptide chain (Beckmann et al., 1997). Posttranslational transport must be fundamentally different, since no ribosome is present. How the driving force is provided in this case is unclear.

Studies in *Saccharomyces cerevisiae* have shown that posttranslational protein transport requires the association of the Sec61p complex with an additional membrane protein complex, the tetrameric Sec62/63p complex, to form a seven-component complex, the Sec complex (Deshaies et al., 1991; Panzner et al., 1995). The Sec complex binds the signal sequence of the translocation substrate (Lyman and Schekman, 1997; Matlack et al., 1997; Plath et al., 1998). Subsequent movement of the polypeptide through the channel requires the additional presence of BiP (also called Kar2p in yeast), a soluble protein of the ER lumen (Vogel et al., 1990). BiP is a member of the Hsp70 family of ATPases and must hydrolyze ATP to translocate polypeptides (Matlack et al., 1997). Translocation requires that BiP interact with the Sec complex via a luminal domain of Sec63p, the J domain, a segment of about 70 amino acids that defines the Hsp70-interacting J protein family (Sadler et al., 1989; Sanders et al., 1992; Brodsky and Schekman, 1993; Scidmore et al., 1993; Lyman and Schekman, 1995; Corsi and Schekman, 1997; Matlack et al., 1997).

Two mechanisms have been proposed by which BiP and the J domain of Sec63p together could provide the driving force for posttranslational translocation. In one, BiP would act as a force-generating motor (Glick, 1995). While simultaneously bound to the import substrate and the J domain, it would undergo a conformational change that would "pull" the polypeptide chain through the channel. Thus, BiP would actively stimulate the forward movement of a substrate. Alternatively, BiP would act as a molecular ratchet (Simon et al., 1992; Schneider et al., 1994). It would bind to the translocating substrate on the luminal side of the channel and prevent it from moving backward. Since movement in the forward direction would not be affected, the bias in movement would eventually result in complete translocation of the substrate. The two proposed mechanisms are not mutually exclusive: a ratcheting mechanism would preserve forward movement into the lumen regardless of whether it was achieved by passive diffusion (Brownian ratchet) or by an active mechanism, such as pulling. Experimentally, the two mechanisms can be tested independently, even if both are operative. Thus, the presence of one could be demonstrated without addressing the existence of the other.

Both models require that BiP bind to the translocation substrate. BiP can indeed interact with a wide variety of peptides. It does so through a peptide-binding pocket that is open when ATP is bound to BiP's N-terminal ATPase domain and closed when ADP is bound (for review, see Bukau and Horwich, 1998). A "lid" domain at the C terminus of the protein may stabilize the closed form of the pocket (Zhu et al., 1996). Peptide binding by BiP is activated by the J domain of Sec63p. In model systems employing recombinant proteins, the J domain stimulates nucleotide hydrolysis (Corsi and Schekman, 1997), and each J domain activates several BiP molecules to trap neighboring peptides with low sequence specificity (Misselwitz et al., 1998).

The motor and ratcheting models have been proposed for mitochondrial protein import (Schneider et al., 1994; Glick, 1995). As in posttranslational ER import, an Hsp70 family member (mtHsp70) in the interior of the organelle is essential. It interacts with Tim44, a protein in the inner mitochondrial membrane (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994), which might act analogously to the J domain of Sec63p. However, Tim44 bears only marginal sequence similarity with a J protein, has not yet been shown to stimulate the ATPase activity of mtHsp70, and may only be required for a subset of imported proteins (Bomer et al., 1998). It is therefore not

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clear whether posttranslational protein transport into the ER and mitochondria occur by the same mechanism. Some experiments suggest a ratcheting mechanism in mitochondrial protein import (Ungermann et al., 1994), but the complexity of intact mitochondria has made a rigorous demonstration difficult.

The availability of well-defined systems that recapitulate posttranslational protein translocation across the ER membrane offers a unique opportunity to test whether BiP acts as a ratchet. Intact ER membranes can be replaced with reconstituted proteoliposomes containing only the purified Sec complex and luminal BiP (Panzner et al., 1995), allowing the function of BiP to be studied in the absence of ER proteins other than the channel components. In addition, a soluble translocation system has been developed (Matlack et al., 1997) in which the role of BiP can be investigated without the complicating effects of a lipid bilayer. Here we have used these systems to demonstrate that BiP and its J partner act as a general and efficient molecular ratchet during posttranslational translocation of the secretory protein prepro-α factor (ppαF). BiP and the Sec complex are the only components required. Furthermore, our data show that a simple Brownian ratchet suffices to achieve translocation.

Results

Transfer of BiP to the Translocating Substrate

A major prediction of a ratcheting model is that BiP binds to the polypeptide chain as it is being translocated through the channel. Cross-linking experiments have shown proximity of BiP to a translocating polypeptide (Sanders et al., 1992), but binding has not been demonstrated. We have used a soluble translocation system (Matlack et al., 1997) to test directly whether BiP is transferred to the substrate. In an initial reaction, a translocation substrate is bound to the yeast Sec complex reconstituted in proteoliposomes. Following solubilization of the membranes, the addition of BiP and ATP induces the movement of the substrate through the channel and its release at the luminal side, as indicated by the fact that release is prevented by a bulky tRNA attached to the C terminus of the substrate (Matlack et al., 1997). We have tested whether BiP is associated with substrate molecules released from the Sec complex as a result of translocation.

The secretory protein ppαF synthesized in an in vitro translation system was chosen as a translocation substrate. To generate a soluble complex of ppαF and Sec complex, the radiolabeled ppαF was incubated with proteoliposomes containing the purified Sec complex (for purity; see Figure 3A, lane 1) and the vesicles solubilized in digitonin. Complex formation was demonstrated by coimmunoprecipitation of ppαF with antibodies against Sec62p, a component of the Sec complex (Figure 1A, lane 4). In the absence of Sec complex, no ppαF was precipitated (lanes 2 and 3). To initiate translocation, purified BiP (see Figure 3A, lane 2) and ATP were added to the soluble complex of ppαF and Sec complex, and the mixture was incubated at 22°C. The reaction was stopped by cooling on ice and adding hexokinase and glucose to rapidly deplete ATP. The reaction time was chosen as a compromise between achieving detectable release of ppαF from the Sec complex and maintaining BiP molecules bound to released ppαF (the association is transient; see below). Immunoprecipitation of the Sec complex demonstrated that under these conditions about 60% of prebound ppαF was released (Figure 1A, lane 5 vs. lane 4). To test whether ppαF released from the Sec complex is associated with BiP, a second round of immunoprecipitation was performed with antibodies against yeast BiP. A significant amount of the released ppαF could be precipitated (lane 17). In a parallel second immunoprecipitation with Sec62p antibodies, much less ppαF was found in the pellet (lane 16), indicating that during the first immunoprecipitation about 90% of the Sec complex was removed. As expected, the amount of ppαF collected with the Sec complex in the second immunoprecipitation was higher in the absence of BiP (lane 13 vs. lane 16). BiP did not interfere with the immunoprecipitation of the Sec complex (data not shown), and without BiP, very little ppαF could be immunoprecipitated by BiP antibodies (lane 14). Together, these data indicate that the BiP-associated ppαF represents molecules released from the Sec complex. Significantly, in the absence of Sec complex, very little ppαF was found to be associated with BiP (Figure 1A, lane 11). Even if ppαF was pretreated with 8 M urea, very little binding to BiP was seen in the absence of the Sec complex (data not shown), demonstrating that BiP alone does not significantly bind to denatured ppαF. BiP binding also required that ppαF was initially bound to the Sec complex; much less binding was seen when ppαF was added to the Sec complex after solubilization (data not shown), conditions that do not allow their interaction (Matlack et al., 1997). Taken together, these data therefore suggest that translocation through the channel is required for ppαF to associate with BiP and that the mere removal of cytosolic proteins or secondary structure from the substrate, which might result from translocation, is not sufficient to induce BiP binding.

To test for a role of the J domain in substrate binding by BiP, we compared the Sec complex from a wild-type strain with that from a yeast strain carrying a mutation in the J domain of Sec63p (sec63-1) that results in a translocation defect in vivo (Rothblatt et al., 1989). The mutant Sec complex binds substrates with the same efficiency as the wild-type complex but is much less active in the release reaction (Matlack et al., 1997). As shown in Figure 1B, it is also much less active in mediating the association of BiP with ppαF. These data indicate that BiP is transferred to the substrate in a reaction that requires its activation by the J domain at the luminal end of the channel.

The association of BiP with ppαF is transient. When BiP and ATP were added to the soluble complex of ppαF and Sec complex and the release reaction stopped at different time points (Figure 1C), ppαF was released from the Sec complex with a half-life of about 2 min (diamonds). At the earliest time point, the majority of the released ppαF (75%–80%) was in a complex with BiP, but with extended incubation periods, the percentage decreased and previously assembled BiP–substrate complexes disassembled (squares). Taken together, these data show that BiP binds to the translocating substrate but does so only transiently.

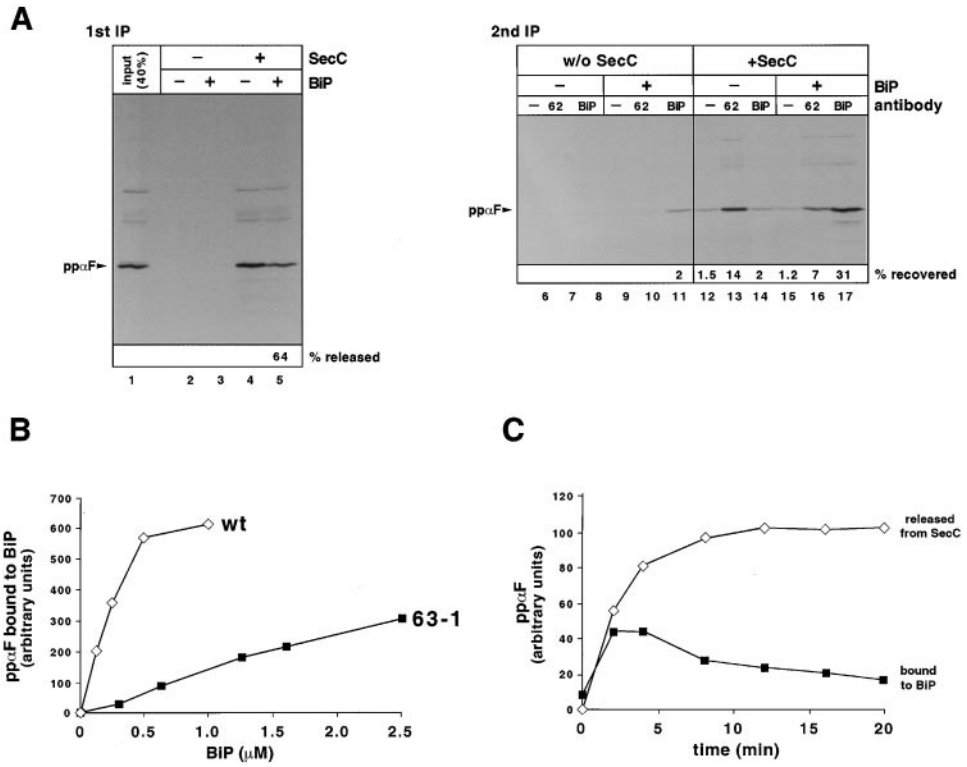

Figure 1. BiP Binds to ppαF during Translocation

(A) In vitro–synthesized, radiolabeled ppαF was bound to Sec complex (SecC) reconstituted into proteoliposomes or incubated with liposomes lacking protein. After solubilization, the samples were incubated with or without 0.55 μM BiP in the presence of ATP for 8 min at 22°C. The reaction was stopped on ice by addition of hexokinase/glucose. The Sec complex together with residually bound ppαF was then collected by immunoprecipitation for Sec62p (left panel). "% released" is the amount of ppαF released by BiP from the Sec complex relative to that originally bound. Released material was subjected to a second round of immunoprecipitation (right panel) with antibodies against BiP, Sec62p (62), or no antibodies (−). "% recovered" gives the percentage of released material recovered in the immunoprecipitations. Lane 1 shows 40% of the input material.

(B) The release reaction was performed as in (A) for 3 min with different BiP concentrations and Sec complex purified from either the wild-type (wt) or the sec63-1 strain, bearing a mutation in the J domain of Sec63p (63-1). The amounts of material recovered in the secondary immunoprecipitation for BiP are plotted.

(C) Release reactions were performed for different time periods with 1 μM BiP. Released material was determined by immunoprecipitation for Sec62p, and material bound to BiP by secondary immunoprecipitation for BiP.

Transfer of Multiple BiP Molecules to a Translocation Substrate

We next determined how many BiP molecules are transferred to a ppαF molecule during the translocation reaction. Proteoliposomes containing Sec complex were incubated with in vitro–synthesized ppαF as before and then floated in a sucrose gradient to remove cytosolic proteins and unbound ppαF before solubilization in digitonin. ppαF subsequently released from the Sec complex in the presence or absence of BiP was analyzed by sucrose gradient centrifugation after removal of the Sec complex by antibodies against Sec62p (Figure 2A). Little ppαF dissociated in the absence of BiP and sedimented as a small and relatively homogeneous species (upper panel, diamonds) almost indistinguishable from free ppαF loaded directly onto a gradient after in vitro synthesis (lower panel). ppαF released by BiP had a heterogeneous size distribution (upper panel, squares) extending from the size of free ppαF upward to approximately 500 kDa (fraction 8). This material does not include residual Sec complex because it did not appear in the absence of BiP (upper panel, diamonds), and in control experiments, ppαF bound to the Sec complex ran as a sharp peak in fraction 8 (data not shown). Consistent with the rapid disassembly of BiP–substrate complexes (Figure 1C), the size of the complexes was largest shortly after initiation of the release reaction (Figure 2B; diamonds) and decreased with time to only slightly larger than the size of free ppαF (squares and circles).

Although in the previous experiment flotation of the vesicles removed the great majority of the cytosolic proteins before BiP was added, we performed further tests to determine if binding of BiP alone is responsible for the size shift of ppαF. For this, the translation mix was brought to 8 M urea and then diluted into a reaction containing proteoliposomes. Under these conditions, all proteins should be stripped off ppαF before it is bound to the Sec complex, and any proteins derived from the translation system that remain after flotation should be denatured and unable to contribute to the size of released complexes. With such a substrate, however, the

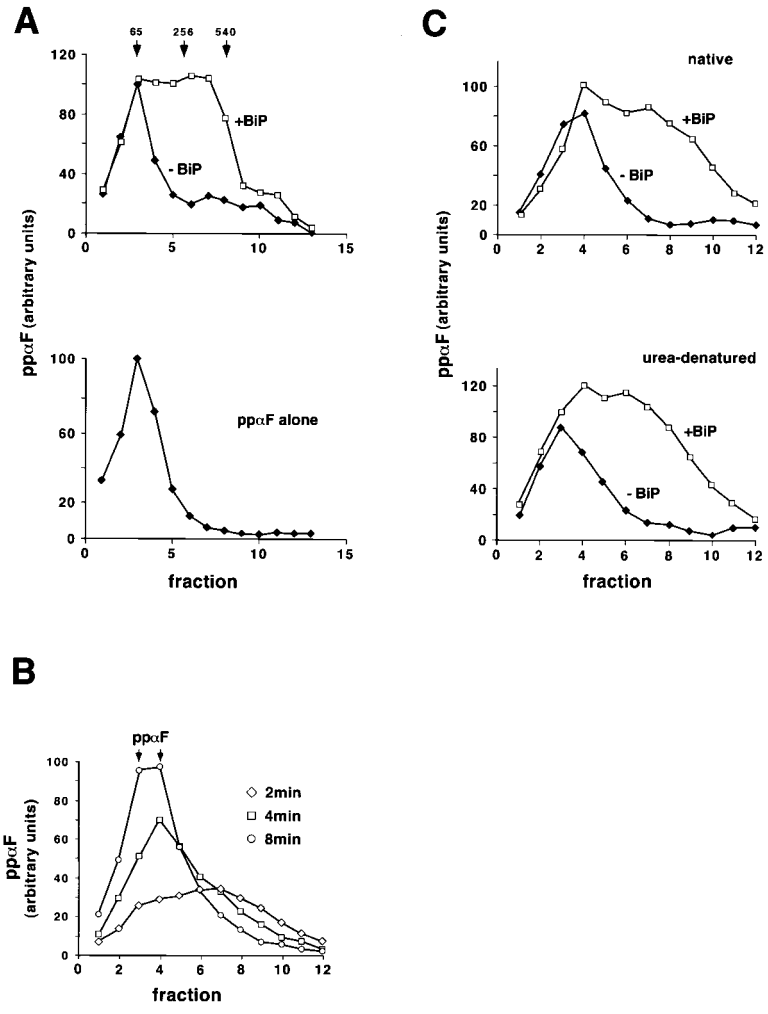

Figure 2. Multiple BiP Molecules Are Bound to a Released ppαF Molecule

(A) Radiolabeled ppαF was bound to Sec complex reconstituted into proteoliposomes. The proteoliposomes were floated to remove unbound material and solubilized. ATP and 2 μM BiP were added and the mixture incubated at 22°C for 2 min to release bound substrate. As a control, BiP was omitted. The reaction was stopped and the Sec complex removed by immunoprecipitation for Sec62p. The supernatants were subjected to sucrose gradient centrifugation (from left to right). Radioactivity in gradient fractions was determined (upper panel). Arrows give the sedimentation positions of marker proteins (bovine serum albumin, catalase, β-galactosidase) and their molecular masses in kilodaltons. The lower panel shows radiolabeled ppαF loaded directly.

(B) As in (A), with variation of the duration of the release reaction. The two peak positions of free ppαF, run in a parallel gradient, are indicated by arrows.

(C) As in (A), except that the ppαF used for the experiments in the lower panel was denatured in 8 M urea.

size distribution of the released complexes was almost indistinguishable from that with native substrate (Figure 2C). Thus, BiP is probably the only component bound to released ppαF.

To further test whether BiP alone is associated with the substrate during translocation, we expressed ppαF in *Escherichia coli*, purified it to homogeneity, and used it to perform translocation reactions composed entirely of purified components (Figure 3A). We first verified that it is indeed transported (Figure 3B). Purified ppαF diluted out of urea was incubated with proteoliposomes containing either BiP alone (lower panel, lanes 12–14), Sec complex alone (lanes 15–17), or both together (lanes 18–20), and translocation was tested by protease protection followed by immunoblotting with antibodies against α factor. Similar to the results with in vitro–synthesized ppαF in the reticulocyte lysate (Figure 3B, upper panel, lanes 1–10), in the presence of both Sec complex and BiP, a significant amount of the added substrate was found to be protected against proteolysis unless detergent was added (lane 19 vs. lane 20). With both the in vitro–synthesized and the purified ppαF, some signal sequence cleavage occurred (lanes 8 and 9 and lanes 18 and 19), caused by a small contamination of the purified Sec complex with signal peptidase. Controls showed that in the absence of either BiP or the Sec complex, little or no protease-protected material occurred with either the ppαF synthesized in reticulocyte lysate (lanes 3 and 6) or the purified ppαF (lanes 13 and 16). We estimate that about 1.3 mol of purified ppαF is transported per mole of correctly oriented Sec71p (a component of the Sec complex). These data establish a system of purified components that reproduces efficient translocation across the ER membrane.

Next, BiP binding to the purified ppαF was assessed as before, using sucrose gradients to analyze the material released from the solubilized Sec complex. The size distribution of material released by BiP was the same as for ppαF in reticulocyte lysate (Figure 3C, lower vs. upper panel). The largest complexes must contain multiple BiP molecules. These data therefore indicate that BiP alone is responsible for the size shift of ppαF. Taking together the results of all sucrose gradient experiments, we estimate that the largest complexes contain six to seven molecules of BiP per ppαF chain of 165 amino acids. This is likely to be the minimum number that interact with a ppαF molecule during its translocation, since dissociation of BiP begins while translocation is still taking place (Figures 1C and 2B). Thus, assuming that each BiP molecule is bound directly to the substrate, a ppαF chain is probably densely covered with BiP molecules immediately after its emergence from the

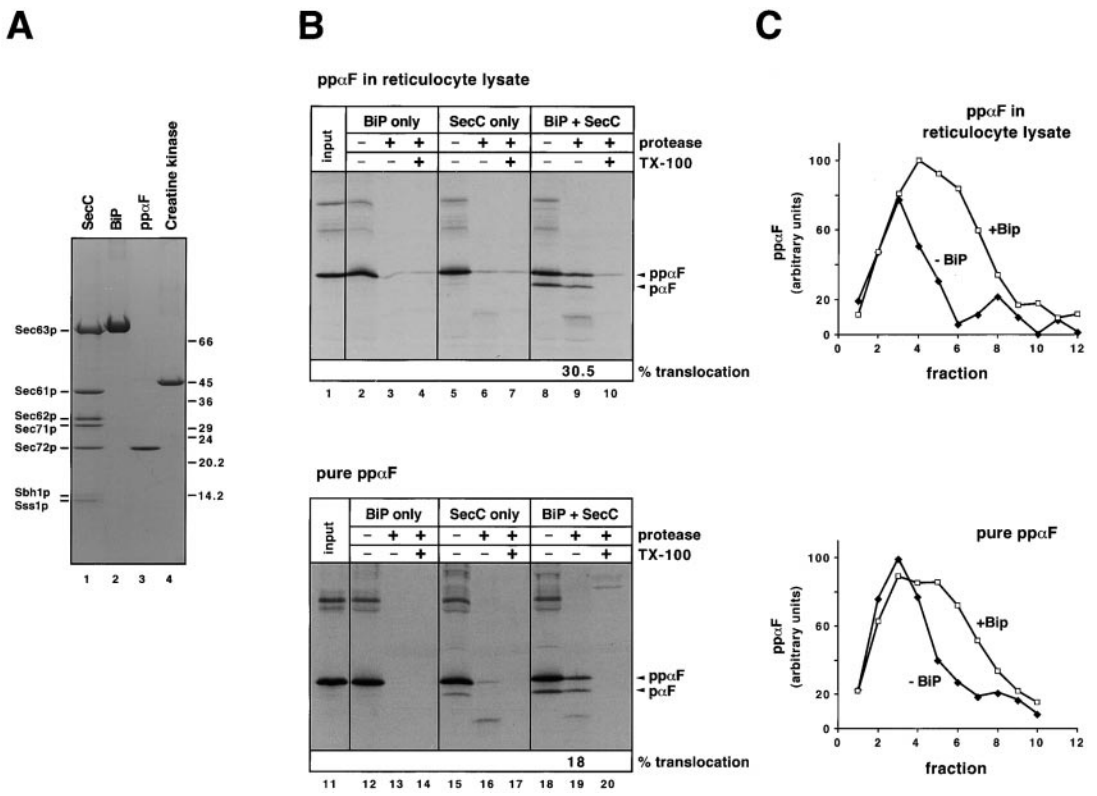

Figure 3. Translocation and BiP Binding with Purified ppαF

(A) ppαF was expressed in *E. coli* and purified. It is shown with the other purified protein components used in translocation reactions. Analysis is by SDS-PAGE and staining with Coomassie blue. The positions of molecular mass markers are indicated on the right in kilodaltons. The individual components of the Sec complex are indicated on the left.

(B) Pure ppαF was incubated with proteoliposomes containing either luminal BiP or Sec complex alone or both together (lower panel). Translocation was assessed by protease treatment in the absence or presence of Triton X-100 (TX-100). "% translocation" is the percentage of protease-protected material relative to the input (shown in lane 11). The upper panel shows a translocation assay with ppαF synthesized in vitro in reticulocyte lysate. pαF indicates the position of pro-αF generated from ppαF by signal sequence cleavage.

(C) Purified ppαF released from the Sec complex in the presence or absence of BiP and ATP was subjected to sucrose gradient centrifugation as in Figure 2A. Each fraction was analyzed by SDS-PAGE and quantitative immunoblotting with antibodies against α factor (lower panel). ppαF synthesized in vitro in reticulocyte lysate was analyzed in parallel (upper panel).

channel. In agreement with the previous demonstration that J-activated BiP has low sequence specificity (Misselwitz et al., 1998), these data also suggest that BiP can bind to many segments of ppαF.

BiP Function Is Required for Vectorial Substrate Movement

We next asked if BiP bound to ppαF could perform the central function of a ratchet, preventing backward movements of the substrate. We first tested whether a partially translocated substrate would slip backward through the channel under conditions in which BiP dissociates. To monitor backsliding, an assay was developed with the following rationale (Figure 4A). If a substrate with a bulky group attached to its C terminus is imported to its maximum extent into proteoliposomes, protease treatment should give a defined fragment corresponding to the piece of the polypeptide chain inside the vesicles (Figure 4A, scheme I). If the substrate slips backward upon dissociation of BiP, protease treatment should result in a heterogeneous mixture of fragments that would be undetectable (Figure 4A, scheme II). Thus, the loss of the specific fragment should be a measure of backsliding.

We first confirmed the validity of the assay. A ppαF substrate with a bulky tRNA at its C terminus (ppαF:tRNA) was generated by in vitro translation of a truncated mRNA coding for a polypeptide chain five amino acids shorter than full-length ppαF. ppαF:tRNA ran as a high-molecular-weight species in SDS gels (Figure 4B, lane 1) and, upon alkaline hydrolysis of the peptidyl–tRNA, was converted to a species slightly smaller than authentic ppαF (lane 2). ppαF:tRNA, released from the ribosomes by the addition of 8 M urea, was incubated at 22°C with proteoliposomes containing Sec complex, luminal BiP, and ATP (lane 5). The reaction mixture was then placed on ice and treated with protease. ppαF:tRNA almost completely disappeared, concomitant with the generation of a protected fragment slightly smaller than the undegraded polypeptide present in the tRNA-associated substrate (lane 6; labeled by a star). The "star" fragment contained more than 50% of the input radioactivity, was insensitive to base hydrolysis, indicating that the tRNA was removed, and did not appear if proteolysis

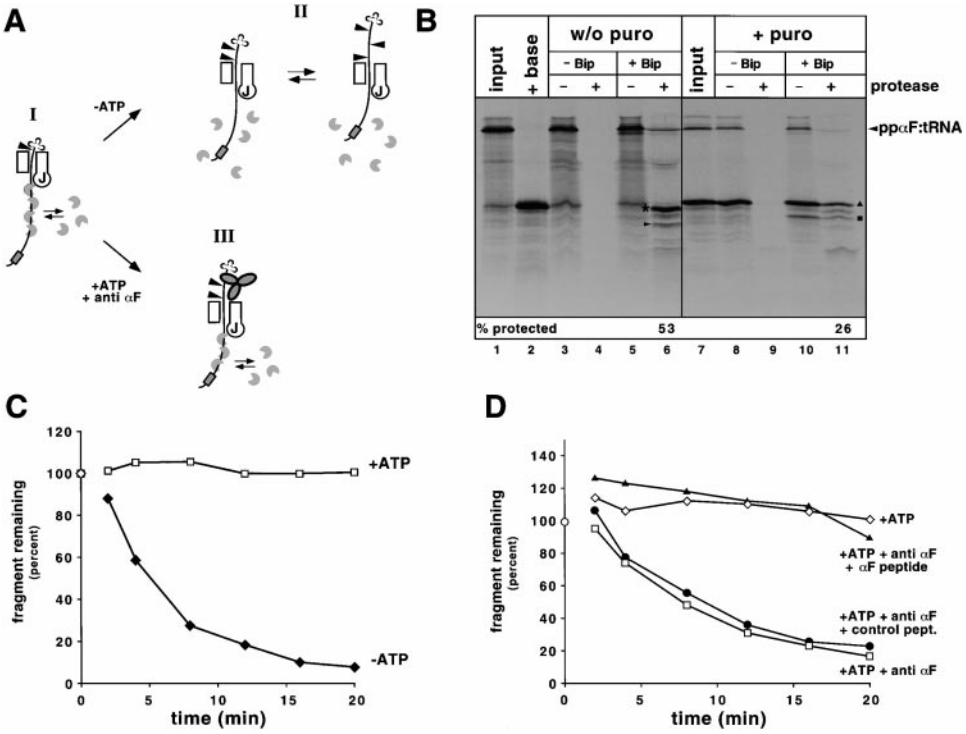

Figure 4. BiP Prevents Backward Movement of Stalled ppαF

(A) Concept of the experiment. A substrate with an attached tRNA is first imported into proteoliposomes containing the Sec complex, BiP, and ATP, resulting in a unique stalled position (scheme I). A single fragment, slightly shorter than the intact polypeptide, will be generated by proteolytic cleavage (arrowhead). Removal of ATP will result in loss of BiP from the substrate, allowing its diffusion back through the channel (scheme II). Heterogeneity of positions of substrate molecules within the channel will prevent one proteolyic fragment from dominating. If backsliding occurs in the presence of ATP, an antibody to the C-terminal domain of the substrate ppαF (anti-αF) will bind and promote the disappearence of the unique proteolytic fragment (scheme III).

(B) Verification of the backsliding assay. A ppαF chain with a tRNA attached (ppαF:tRNA) was generated by in vitro translation of a truncated mRNA coding for a polypeptide five amino acids shorter than full-length ppαF. The presence of the tRNA was demonstrated by the size shift following base hydrolysis (lane 2 vs. lane 1). ppαF:tRNA, released from the ribosomes by 8 M urea, was imported into Sec complex–containing proteoliposomes with or without BiP and ATP in their interior (lanes 3–6). As a control, the tRNA was removed from ppαF:tRNA by puromycin treatment before addition of 8 M urea (ppαF:puro; lanes 7–11). The samples were proteolyzed as indicated and subjected to SDS-PAGE under conditions that preserve the tRNA–peptide bond. The star indicates a fragment derived from the stalled translocation substrate, as predicted in scheme I of (A). The arrowhead indicates a fragment presumably differing from the star fragment by the absence of the signal sequence. The triangle and square indicate ppαF:puro and its signal sequence–cleaved form, respectively. "% protected" gives the amount of star fragment or ppαF:puro relative to the input material.

(C) Backsliding upon ATP depletion. tRNA-associated ppαF was imported to produce a stalled translocation substrate as in (B). Either hexokinase and glucose (−ATP) or an ATP-regenerating system (+ATP) was then added (zero time point), and aliquots were proteolyzed on ice at times thereafter. Plotted is the amount of the star fragment formed (see [B]), with 100% being the amount at the zero time point.

(D) Backsliding in the presence of ATP. tRNA-associated ppαF was imported to produce a stalled translocation substrate as in (B). Antibodies against α factor (anti-αF) were added (zero time point) and the amount of star fragment determined after proteolysis. Controls were performed with antibodies preincubated with the α factor peptide (αF peptide) or with a peptide containing the same amino acid composition but a scrambled sequence (control pept.).

was performed in the presence of detergent (data not shown). Thus, as expected, the tRNA of the stalled substrate was located on the cytoplasmic side of the vesicles, immediately adjacent to the membrane, while the N terminus of ppαF was inside the vesicles (Figure 4A, scheme I). Among the additionally generated minor bands, one probably corresponds to the signal sequence–cleaved form of the fragment (lane 6, arrowhead), but the origin of the others is unclear. None of the fragments were seen in the absence of BiP (lanes 3 and 4) or the Sec complex (not shown). As a further control, we tested ppαF whose tRNA was removed by puromycin treatment and that therefore should be fully translocated into the lumen of the vesicles (lanes 7–11). The puromycin reaction resulted in most of the label being present in a small species (ppαF:puro), although some ppαF:tRNA remained (lane 7 vs. lane 1). Upon incubation with proteoliposomes containing Sec complex, BiP, and ATP, ppαF:puro and its signal sequence–cleaved form became fully protected against proteolysis (lane 11 vs. lane 10, triangle and square, respectively). A small amount of what is probably the star fragment was also generated (between the triangle and square), most likely from the residual ppαF:tRNA. No translocation was seen in the absence of BiP (lane 8 vs. lane 9) or the Sec complex (data not shown). Taken together, these data show that the appearance of the star fragment is indicative of the ppαF species stalled in the channel by the attached tRNA.

We used the star fragment to test whether backsliding

of stalled ppαF:tRNA occurs when BiP dissociates. ppαF:tRNA was first imported into proteoliposomes containing Sec complex, BiP, and ATP, and then hexokinase and glucose were added to deplete ATP. The vesicles are sufficiently permeable to ATP to allow its rapid depletion (see also Panzner et al., 1995), preventing further BiP binding while allowing dissociation of BiP from the substrate to occur. Following ATP depletion, the samples were incubated at 22°C for different time periods, placed on ice, and treated with protease. With time, less of the protected star fragment was generated (Figure 3C, filled symbols), indicating that backsliding of the substrate indeed occurred. Control experiments without proteolysis demonstrated that the amount of ppαF:tRNA precipitable with BiP antibodies after solubilization decreased with time (data not shown). In contrast, in the continuous presence of ATP, the amount of protected fragment generated remained constant (open symbols). In neither case did the total amount of substrate change in the absence of protease, nor did ATP depletion render fully translocated ppαF:puro sensitive to proteolysis (data not shown). These results indicate that in the absence of bound BiP the substrate can move backward passively, indicating that the channel itself cannot "hold" the substrate; the continuous action of BiP is required to maintain the translocation substrate in its stalled position and thus preserve the extent of translocation already achieved.

We next asked if transient backward movements occur when ATP is present (Figure 4D). ppαF:tRNA was imported into proteoliposomes containing Sec complex, BiP, and ATP as before, and an antibody directed against α factor, a domain repeated four times at the C terminus of ppαF (see Figure 6A), was added to the exterior of the proteoliposomes. The antibody induced the disappearance of the star fragment (squares), indicating that it must have gained access to segments of molecules that had previously been translocated into or through the channel. Thus, backsliding of the substrate must occur even when ATP is present. Controls demonstrated that presaturation of the antibody with an α factor peptide blocked its effect (Figure 4D, filled triangles), in contrast to a control peptide with an identical amino acid composition but scrambled sequence (filled circles). These data also exclude the possibility that backsliding under these conditions is due to ATP depletion. Taken together, these results support a ratcheting mechanism: transient backward movements of the substrate do occur during translocation and are minimized by the binding of BiP at the luminal side of the membrane.

A BiP Mutant Impaired in Ratcheting

If BiP acts as a ratchet during translocation, then the length of time that it spends bound to a substrate is crucial; a BiP mutant with an increased rate of dissociation from the substrate should be less effective in preventing backward movements and thus be less efficient at promoting translocation. We used a truncation mutant containing both the peptide-binding and ATPase domains of BiP but lacking the C-terminal lid domain (BiPΔlid). BiPΔlid has an increased dissociation rate from a synthetic peptide in its ADP form, the form in which BiP is ultimately bound to the substrate, but its rate of association in ATP is almost identical to that of wild-type BiP (Misselwitz et al., 1998; unpublished results).

We first examined the BiPΔlid mutant in the soluble translocation system, in which the release of prebound substrate from the Sec complex is determined (see Figure 1A). BiPΔlid was active in releasing ppαF from the solubilized Sec complex, but higher concentrations than with wild type were required (Figure 5A, upper panel). At high concentrations of BiPΔlid, some ppαF could be coprecipitated with BiP antibodies, but coprecipitation was much less efficient than with wild-type BiP, despite complete recovery of BiPΔlid (data not shown). These data are thus consistent with the expected higher dissociation rate of the substrate–BiP complexes. As a control, we tested a mutant that contained only the ATPase domain and lacked both the peptide-binding and lid domains; it proved inactive in the release reaction (Figure 5A). Interestingly, the curve of ppαF release from the Sec complex displayed a clear shoulder at low concentrations of BiPΔlid (Figure 5A, upper panel), suggesting that more than one BiP molecule is required for efficient release of ppαF from the Sec complex. A slight sigmoidicity was also seen with wild-type BiP at very low concentrations (Figure 5A, lower panel, inset; data not shown).

Similar results were obtained with another posttranslational translocation substrate, proOmpA (Figure 5A, lower panel). Again, significantly higher concentrations of BiPΔlid were required to achieve the same extent of release as with wild-type BiP, and sigmoidicities were observed with both the mutant (Figure 5A, lower panel) and wild-type BiP (inset). Compared to ppαF, proOmpA required an approximately 2-fold higher concentration of BiP for half-maximal release (Figure 5A). Wild-type BiP and BiPΔlid showed similar differences when they were reconstituted into proteoliposomes containing the Sec complex, and translocation was assayed with either ppαF or proOmpA (data not shown). Taken together, these results indicate that the BiPΔlid mutant behaves in translocation as a ratcheting mechanism would predict for a molecule with an enhanced rate of dissociation: since it spends less time bound to the translocation substrate, higher concentrations are required to prevent backward movements.

To confirm that BiPΔlid is impaired as a ratchet we used the backsliding assay. ppαF:tRNA was imported into proteoliposomes containing the Sec complex, ATP, and high concentrations of either wild-type BiP or BiPΔlid, and backward movements were assessed by the decrease in the amount of star fragment generated by protease treatment (Figure 5B). Backsliding occurred in the absence of ATP and was much faster with BiPΔlid than with wild-type BiP. In the presence of ATP, the amount of star fragment remained constant. The difference in slip-back rates between mutant and wild-type BiP indicates that with the latter, backward movements of the substrate within the channel are limited by the rate at which BiP dissociates and not by interactions between the substrate and the channel or by the rate of ATP depletion.

A Brownian Ratchet Promotes Translocation

We next tested whether protein translocation can occur by Brownian ratcheting, in which forward movement

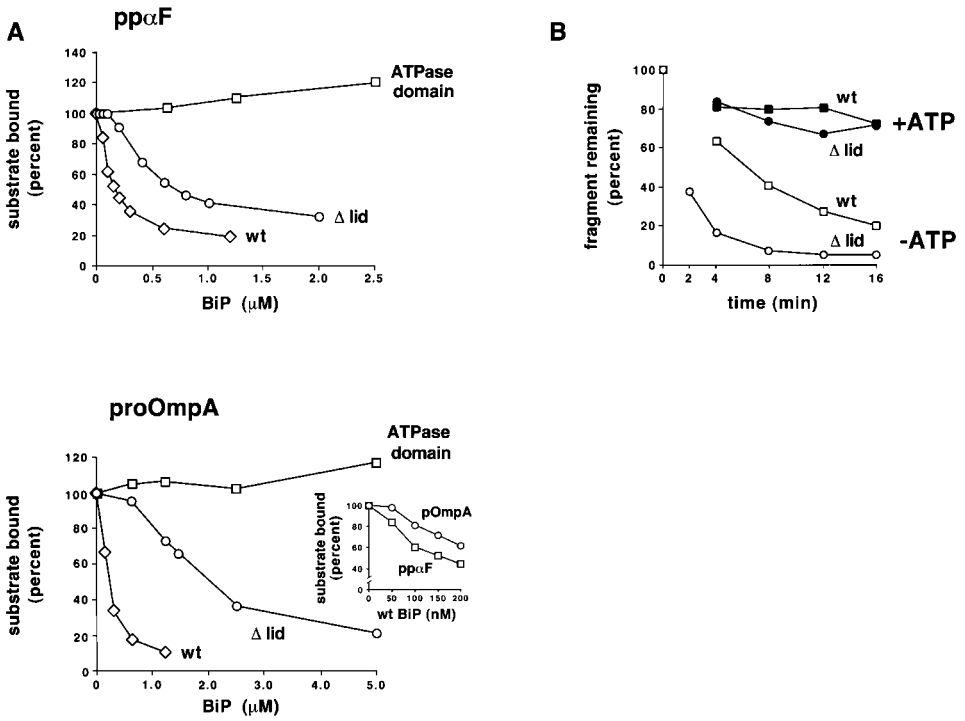

Figure 5. A Ratcheting Mutant of BiP

(A) ppαF or proOmpA bound to the soluble Sec complex was released for 10 min with different concentrations of wild-type (wt) BiP or truncated versions lacking either the lid (Δlid) or the entire peptide-binding domain (ATPase domain). The percentages of substrate remaining associated with the Sec complex are plotted. Note the difference in the scales of the horizontal axes in the upper and lower panels. The inset in the lower panel shows release of ppαF and pOmpA at low concentrations of wt BiP.

(B) ppαF:tRNA was imported into proteoliposomes containing Sec complex, ATP, and wt or mutant BiP lacking the lid domain (Δlid). Backsliding of the substrate was tested in the presence or absence of ATP, as in Figure 3C.

of the polypeptide chain occurs by simple diffusion. Specifically, we asked whether ppαF is translocated if BiP is replaced in the lumen by nonphysiological binding partners of the translocation substrate. We used as binding partners antibodies directed against different regions of ppαF (Figure 6A), each of which efficiently immunoprecipitates the protein (data not shown). In vitro–synthesized ppαF was incubated with proteoliposomes containing the Sec complex in the membrane and various combinations of antibodies in their interior, and translocation was assessed by protease protection in the absence or presence of detergent (Figure 6B). With no antibodies, or with antibodies directed against segments in the middle or at the C terminus of ppαF (antibodies 2 and 3), very little translocation was seen (Figure 6B, lanes 3, 6, and 9; for quantitation, see Figure 6C). However, with antibody 1, directed against the region immediately following the signal sequence, significant levels of translocation were observed (Figure 6B, lane 12). The additional presence of the other antibodies resulted in a reproducible further increase (lanes 15 and 18). When the antibodies were added to the outside of the vesicles, no protease-protected material was seen (data not shown). Although the antibody-driven translocation reaction is somewhat less efficient than that with BiP and ATP (up to 23% vs. 50%–70% under optimal conditions [data not shown]), it is clear that translocation can occur with neither input of energy in the form of ATP nor interaction of the ratcheting molecule with the channel. Thus, passive sliding of ppαF through the channel, coupled with its binding to antibodies in the interior of the vesicles, suffices to achieve its complete transport through the membrane.

Discussion

Our results show that a simple Brownian ratchet is sufficient to provide the driving force for translocation. All that is required is the channel-forming Sec complex and a binding partner for the translocation substrate on its luminal side. Although any binding partner would drive translocation, the physiological partner BiP functions as a general and efficient ratchet. Multiple BiP molecules are transferred to each substrate molecule during translocation, and BiP binding is dependent on an interaction with the J domain of Sec63p. These data suggest that BiP associates with essentially any segment of a translocating polypeptide chain at the luminal end of the channel where the J domain is located. The polypeptide chain was found to slide through the channel in either direction, and transient backward diffusion occurred even under conditions that promote translocation. Binding of BiP minimized such unproductive backward movements of the polypeptide chain, and BiP's effectiveness as a ratchet was crucially dependent on the time that it spent bound to the substrate. Our data indicate that BiP has

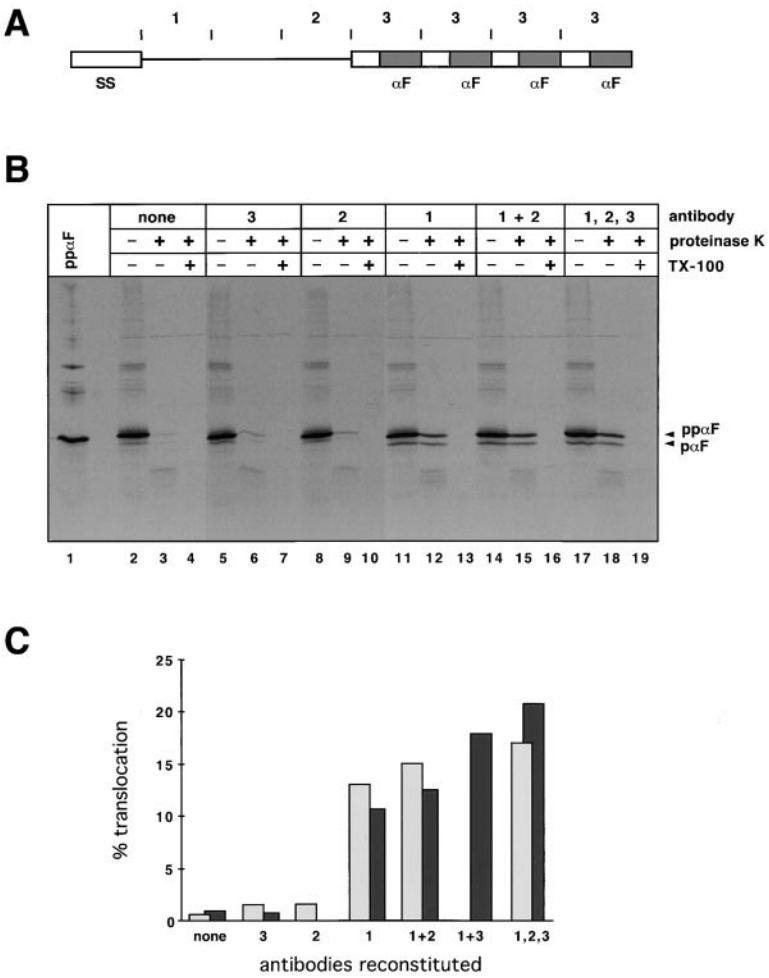

Figure 6. Antibodies against ppαF Can Drive Translocation

(A) Regions of ppαF against which antibodies are directed. ss and αF indicate the signal sequence and the α factor repeat domain.

(B) In vitro–synthesized ppαF was incubated with proteoliposomes containing the Sec complex in the membrane and various combinations of antibodies in their interior. Translocation was assessed by treatment with proteinase K in the absence or presence of Triton X-100. Lane 1 shows the input material. pαF indicates the position of pro-αF generated from ppαF by signal sequence cleavage.

(C) Quantitation of the experiment in (B) (light columns). Dark columns are a different experiment.

several properties that make it superior to other potential ratcheting molecules: (1) it most likely binds immediately at the luminal end of the channel, where it could most effectively prevent backward movements of the substrate; (2) it has low sequence specificity, which allows it to bind to many sites on a given polypeptide and would allow it to transport a wide range of substrates; and (3) its interaction with the substrate is transient, being bound long enough to promote translocation but dissociating quickly enough to allow subsequent folding or modification reactions. BiP is probably also the only ratchet operative during translocation; because it coats a substrate, other molecules would be prevented from participating.

A specific model for how posttranslational translocation may occur is shown in Figure 7. In the first step, the translocation substrate is bound to the Sec complex by virtue of its signal sequence (shaded box) and is inserted into the channel (Figure 7, scheme I; Matlack et al., 1997; Plath et al., 1998). The substrate is probably bound as a loop with a small portion in the luminal space (Shaw et al., 1988; Mothes et al., 1994; Plath et al., 1998). BiP does not seem to be required to open the channel or transfer the polypeptide into it, at least not in the reconstituted system with ppαF as the substrate, since ppαF could gain access to antibodies in the lumen of proteoliposomes in the absence of BiP. Consistent with the assumption that the segment immediately following the signal sequence would be the first to appear in the

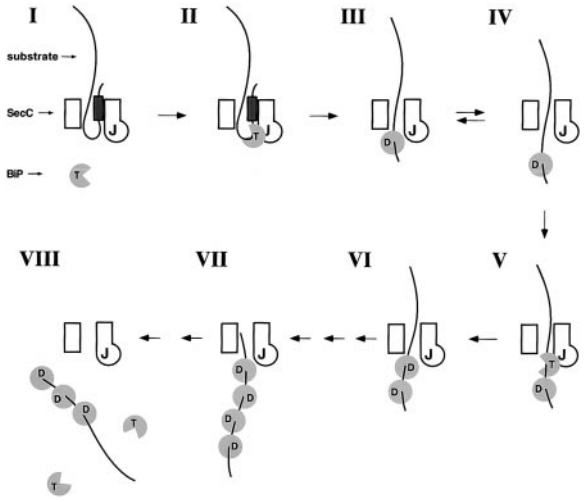

Figure 7. A Ratchet Model for Posttranslational Protein Translocation into the ER

See text for details.

lumen (Figure 7, scheme I), antibodies directed against this domain were most effective in promoting translocation; more C-terminal regions would have to diffuse farther to become accessible to luminal antibodies.

The BiP ratchet would be initiated by a transient interaction of BiP in its ATP form with the J domain of Sec63p (Figure 7, scheme II). The J domain activates BiP for peptide binding. It induces rapid hydrolysis of the nucleotide, converting the open peptide-binding pocket of BiP–ATP into the closed pocket of BiP–ADP and resulting in the ADP form bound to the translocating polypeptide chain (scheme III). J-activated BiP is very short lived and binds peptides with low specificity (Misselwitz et al., 1998), allowing it to bind to essentially any segment of the substrate close to the luminal end of the channel. Attachment of a BiP molecule would prevent the bound segment of the substrate from reentering the channel but would not hinder forward movements (Figure 7, schemes III and IV). The polypeptide may move back and forth (double arrow), but once enough has moved into the lumen, the next BiP molecule would bind by the same mechanism (schemes V and VI) and this process would be repeated until the polypeptide chain is entirely translocated (scheme VII). The presence of multiple BiP molecules on a substrate increases the efficiency of the ratcheting mechanism (Simon et al., 1992). BiP would dissociate from the substrate following nucleotide exchange (Misselwitz et al., 1998); binding of ATP would reopen the peptide-binding pocket and release the substrate (scheme VIII). It should be noted that in our in vitro experiments most substrate molecules retain their signal sequence; its dissociation from the Sec complex appears to be spontaneous, allowing it to be translocated along with the rest of the substrate.

Although our antibody experiments show that ATP is not required for a ratchet per se, the ATPase cycle is essential for BiP to function as a ratchet. J-activated ATP hydrolysis is required for efficient binding to the substrate, to localize binding at the end of the channel, and to make binding largely sequence independent. The ATPase cycle also makes BiP's association with the substrate a steady-state—rather than an equilibrium—condition, ensuring eventual release of the ratcheting molecules.

It is possible that the driving force for posttranslational protein translocation is provided entirely by BiP acting as a Brownian ratchet, since significant levels of translocation were achieved even with antibodies as luminal binding partners of the substrate. BiP's greater efficiency can be explained by its ability to bind to more segments of the substrate and by the localization of its binding at the end of the channel. And, although antibodies may bind the substrate tighter, BiP clearly binds to the substrate long enough to act as an efficient ratchet, since even fully translocated ppαF molecules are still associated with several BiP molecules. Thus, there appears to be no necessity to invoke a mechanism by which forward movement would be achieved by an active mechanism. On the other hand, we cannot exclude that BiP actively "pulls" on the substrate in addition to its function as a ratchet: after generating force while bound to both the J domain and the substrate, BiP would remain bound only to the substrate, acting as a ratchet to preserve the forward movement caused by the power stroke. It is possible that force generation by BiP would be required if the substrate was folded into a stable conformation on the cytosolic side of the membrane or if cytosolic proteins were strongly bound, as has been proposed in the case of mitochondrial protein import (Glick, 1995). However, the rate of mitochondrial protein import does not seem to exceed the spontaneous rate of unfolding of a substrate on the outside of mitochondria (Gaume et al., 1998; but see Matouschek et al. [1997] for a different view). In addition, posttranslational substrates may have been selected to have relatively loosely folded domains; the parallel cotranslational pathway may have evolved precisely because it is independent of the folding properties of a substrate. Although most of our experiments were performed with ppαF, our data with proOmpA are consistent with a ratchet also operating during its translocation.

Our data show that ppαF can diffuse in either direction within the channel, suggesting that the channel itself is a passive conduit for polypeptides. This concept is supported by the fact that the same Sec61p channel is used in cotranslational translocation, in which the polypeptide appears to diffuse through the channel. Nevertheless, the channel may not be just a rigid pore, and its walls could make contact with the polypeptide chain passing through it.

Because the channel itself is passive, it could be used for transport in either direction. It is the position of the J domain of Sec63p at the luminal end of the channel that determines the directionality of posttranslational protein transport into the ER. Cytosolic Hsp70 cannot enter a translocation "tug of war" with BiP because it does not have a cytosolically disposed J partner associated with the channel and therefore cannot bind to the substrate. However, it is conceivable that under certain conditions, rather than associating with the Sec62/63p complex, the Sec61p channel could associate instead with a cytosolic J protein. This would allow cytosolic Hsp70s to be recruited for a retrograde ratcheting process. Such could be the case, for example, in the retrograde transport of proteins from the ER for cytosolic destruction by the proteasome (for review, see Kopito, 1997).

BiP has also been proposed to function in cotranslational protein translocation (Nicchitta and Blobel, 1993; Brodsky et al., 1995; Hamman et al., 1998). However, in a defined reconstituted system, neither BiP nor the Sec62/63p complex, containing the J domain critical for the operation of a ratchet, are required (Gorlich and Rapoport, 1993). Thus, cotranslational translocation would have to use BiP in a different way or perhaps convert to the posttranslational mode during the translocation of particular substrates.

A ratcheting mechanism involving an Hsp70 may also provide the driving force for protein import into mitochondria (Schneider et al., 1994). It appears that a passive channel that binds the substrate on one side and allows the substrate to diffuse through it and encounter a binding partner on the other side may be a simple and widespread translocation mechanism.

Experimental Procedures

Purification of ppαF

ppαF with six His residues at the C terminus was expressed in *E. coli* after induction with isopropyl-β-D-thiogalactopyranoside. Cells

were lysed in 6 M guanidine-HCl, 100 mM sodium phosphate, 10 mM Tris/HCl (pH 8.0), and the extract applied to a Ni-NTA column. Elution was in 8 M urea, 100 mM sodium phosphate, 10 mM Tris/HCl (pH 4.5). Eluted material was run on a C18 HPLC column in 0.1% trifluoroacetic acid with a gradient of 0%–80% acetonitrile. Dried peak fractions were dissolved in 60% acetonitrile.

Preparation of Antibodies and BiP Mutants

Rabbit antisera against purified His-tagged BiP were affinity purified as described for Sec62p antibodies (Panzner et al., 1995). Rabbit antisera against purified ppαF were affinity purified against the immobilized peptides KNTTTEDETAQIPAEAVIG, CNTTIASIAAKEEGV SLD, and CEAWHWLQLKPGQPMYKRE. BiP's ATPase domain and BiPΔlid (amino acids 42–552) were produced essentially as described (Misselwitz et al., 1998).

Preparation of In Vitro–Translated Translocation Substrates

Preparation and translation of full-length mRNAs were as in Panzner et al. (1995). ppαF with a C-terminal tRNA (ppαF:tRNA) was made by cutting plasmid pSP65–ppαF with NciI and transcribing with SP6 polymerase. Translation was at 30°C in reticulocyte lysate with 0.74 μCi/ml [^{35}S]methionine. After dilution with cold buffer A (50 mM HEPES/KOH [pH 7.5], 150 mM potassium acetate [KOAc], 5 mM magnesium acetate [MgOAc], 2 mM dithiothreitol [DTT]), samples were centrifuged through 450 μl of buffer A at 500 mM sucrose for 1 hr at 75,000 rpm and 2°C in a Beckman TL100.3 rotor. Pellets were resuspended in buffer A at 250 mM sucrose (buffer B). Puromycin treatment was at 1 mM in buffer B with 267 mM KOAc for 10 min on ice and 30 min at 35°C.

Release Reactions

Sec complex (SecC) was affinity purified and reconstituted as in Panzner et al. (1995). Vesicles were used without centrifugation. Binding of substrates to reconstituted SecC, solubilization, and release by BiP were as described (Matlack et al., 1997).

Slip-Back Reactions

SecC proteoliposomes were produced with 1.9 μM wild-type or 10 μM mutant BiP and 2 mM ATP, ~0.2 mg/ml creatine kinase (CK), 10 mM creatine phosphate (CP), and 2–2.5 mM MgOAc included during reconstitution. Vesicles were diluted 50-fold with buffer A at 3% glycerol and ATP, CK, and CP present. Samples were centrifuged for 45 min at 2°C and 75,000 rpm in a Beckman TL100.3 rotor and the pellets resuspended to the original volume in buffer C (buffer A plus 15% glycerol) with 2.5 mM ATP, 0.25 mg/ml CK, and 5 mM CP.

tRNA- and puromycin-associated substrates were brought to 8 M urea. Import reactions were for 5 min at 22°C with 10% (v/v) urea-denatured substrate in buffer C at 1 mM ATP, 0.1 mg/ml CK, and 5 mM CP. For slip-back after ATP depletion, one aliquot was brought to 0.1 U/μl hexokinase (HK) and 20 mM glucose, and another remained in ATP. After incubation at 22°C, aliquots were brought to 0.5 mg/ml proteinase K (prK) and placed on ice for ~50 min. The samples were precipitated with 10% trichloroacetic acid (TCA) and heated briefly at 32°C in SDS sample buffer at pH 6.8. Quantitation after SDS-PAGE was with a phosphorimager. For slip-back in ATP, aliquots of import reactions were mixed with 0.2–0.5 mg/ml of affinity-purified antibodies against the α factor repeat region of ppαF. Control reactions included the peptide KREADAEAWHWLQLKPGQ or the control peptide RKAEADAEHWLWLQPKQG (final A_{280} 0.51).

Translocation Reactions

Translocation reactions contained 40% proteoliposomes with SecC, BiP, an energy-regenerating system, and 50% buffer C. Either in vitro–synthesized ppαF or 0.35 μg/ml pure ppαF, both in 8 M urea, was added and the incubation performed at 22°C for 45 or 15 min, respectively. Samples were proteolyzed on ice with 0.5 mg/ml prK with or without 1% Triton X-100 for 1 hr, and then 2 mM PMSF was added for 10 min before SDS-PAGE. For pure ppαF, analysis was by immunoblotting with α factor antibodies and ^{35}S-labeled secondary antibodies.

Antibody-Driven Translocation Reactions

SecC proteoliposomes containing 100 μg/ml of each antibody were treated with protein A–Sepharose beads for 1 hr at 4°C to remove unincorporated antibodies. Vesicles (14.4 μl) were mixed with 3.6 μl of ppαF translated in reticulocyte lysate and the mixture incubated at 22°C for 1 hr. Further analysis was as in "Translocation Reactions."

Sucrose Gradient Centrifugation

SecC proteoliposomes with bound ppαF were floated for 1 hr at 55,000 rpm and 2°C in a Beckman TLS55 rotor. Floated vesicles were solubilized at 12% glycerol and 1% digitonin. BiP (2 μM) and an energy-regenerating system were added. After 2 min at 22°C, the reaction was placed on ice and 0.1 U/μl HK and 10 mM glucose added. After immunoprecipitation with Sec62p antibodies, 20 μl of the supernatant was loaded on a 255 μl 9%–15% (w/v) sucrose gradient in buffer A with 1% digitonin and 0.5 mM ADP and centrifuged in a Beckman TLA100 tube for 2 hr at 55,000 rpm and 2°C in a Beckman TLS55 rotor. Radioactivity in 20 μl fractions was determined by scintillation counting. Pure ppαF was analyzed by immunoblotting.

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